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Prevalence of Germline Mutations in Patients with Pheochromocytoma or Abdominal Paraganglioma and Sporadic Presentation: A Population-Based Study in Western Sweden

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Abstract
Background Germline mutations in the susceptibility genes *RET*, *SDHB*, *SDHD*, and *VHL* have been reported in 7.5–24% of patients with pheochromocytoma (Pheo) or paraganglioma (PGL) and sporadic presentation. The purpose of the present study was to establish population-based data on the frequency of germline mutations in patients with apparently sporadic Pheo or abdominal PGL in Western Sweden.
Methods From the Swedish National Cancer Registry, all patients with Pheo or PGL in Western Sweden (population 1.72 million) registered between 1958 and 2009 were identified ($n = 256$). Patients were characterized using register data, hospital records, and clinical interviews. All living patients with Pheo or abdominal PGL and sporadic presentation ($n = 81$) were invited to genetic screening; 71 patients accepted. Germline mutations were investigated by using direct sequencing for point mutations in *RET*, *SDHB*, *SDHD*, and *VHL*, and multiplex ligation-dependent probe amplification for gross deletions in *SDHB*, *SDHC*, *SDHD*, and *VHL*. Plasma or urinary metanephrines and/or urinary catecholamines were used for biochemical follow-up.
Results The prevalence of germline mutations was 5.6%. Mutations were only seen in *RET* ($n = 1$) and *SDHB* ($n = 3$). Notably, in the patients with *SDHB* mutations, no malignant phenotype was observed during a mean follow-up of 23.3 years.
Conclusions The frequency of germline mutations in patients with apparently sporadic Pheo and abdominal PGL in Western Sweden was lower than in previous studies. Variations in reported frequencies of germline mutations in patients with clinically sporadic Pheo/PGL may reflect geographical differences or patient selection.

Introduction

The list of known genes causing hereditary pheochromocytoma (Pheo) or paraganglioma (PGL) is expanding and includes *NF1* in neurofibromatosis type 1 (NF1), *RET* in multiple endocrine neoplasia syndrome type 2 (MEN2), *VHL* in Von Hippel–Lindau syndrome (VHL), succinate dehydrogenase subunit genes *SDHB*, *SDHC*, and *SDHD* and the succinate dehydrogenase complex assembly factor 2 gene *SDHAF2* in familial paraganglioma syndromes PGL4, PGL3, PGL1, and PGL2, respectively. Recently mutations in *PHD2* [1], *SDHA* [2], *TMEM127* [3], *Kif-1Bβ* [4], and *MAX* [5] have been described in association with Pheo/PGL. Since Neumann et al. [6] in 2002 reported 24% germline mutations in *SDHB*, *SDHD*, *RET*, and *VHL* in patients with clinically nonsyndromic Pheo/PGL, genetic screening in these patients has attracted considerable interest. An algorithm for testing based on clinical risk factors (family history or age <35 years, extra-adrenal, bilateral or malignant tumours) was published in 2006 [7],

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and several modifications have already been suggested [8–10]. In cohorts with Pheo/PGL and sporadic presentation published after 2002, the frequency of germline mutations have ranged from 7.5 to 19.1% [8–12]. The wide variation may represent geographical differences and/or patient selection, and population-based studies are needed.

To establish population-based data on the frequency of germline mutations in patients with apparently sporadic Pheo or abdominal PGL in Western Sweden, we have offered genetic screening to all living patients from our region registered in the National Cancer Registry (NCR) from 1958 to 2009.

Patients and methods

Setting

The western healthcare region in Sweden comprises the region of Västra Götaland and the municipalities Varberg, Falkenberg, and Kungsbacka, with a total population (June 2008) of 1.72 million inhabitants.

Data sources

National Cancer Registry

Mandatory reporting of Pheo and PGL to the NCR started in 1958. The reporting frequency is high; approximately 96% of cases were reported to the NCR according to a recent survey [13]. From the NCR, all patients in the western healthcare region with a diagnosis of Pheo or PGL were identified. Search terms were: ICD-7 localization codes 195.0 (adrenal medulla) and 195.7 (paraganglia); morphology codes 441 (benign Pheo/PGL) and 446 (malignant Pheo/PGL or malignant neuroendocrine tumor). Only patients with histopathologically verified disease were included.

Hospital records and clinical interviews

From register data, hospital records and clinical interviews information on diagnosis, age at diagnosis, presentation (sporadic or hereditary/syndromic), tumor location and secretory profile, and metastatic or recurrent disease was collected. Sporadic presentation was defined as a negative family history and absence of syndromic lesions associated with MEN2, NF1, or VHL at diagnosis. Malignancy was defined as presence of metastases at the time of diagnosis or during follow-up. To distinguish metastatic from multifocal disease, a diagnosis of metastatic disease required that chromaffin tissue was present at a site where chromaffin tissue is not otherwise found [14]. Because criteria for malignancy in Pheo and PGL have changed over time, histopathological reports were reviewed and in selected cases specimens were reanalyzed to update diagnoses to the current definitions. Plasma or urinary metanephrines and/or urinary catecholamines were used for biochemical follow-up.

Eligibility criteria

All patients with Pheo or abdominal PGL and sporadic presentation were invited to genetic screening, which started in spring 2006. All participating patients gave oral/written consent. The study was approved by the Regional Ethical Review Board in Gothenburg (registration number 652-06).

Genetic screening

Based on the presumed frequency of findings stepwise genetic testing was performed in the following order: (1) Sanger sequencing for point mutations in *SDHB* (exon 1–8), *SDHD* (exon 1–4) and *VHL* (exon 1–3); (2) In patients with negative sequencing results in *SDHB*, *SDHD*, and *VHL* sequencing of *RET* (exons 10, 11, 14, and 16); (3) In patients negative after testing for *RET*-mutation the presence of deletions in *SDHB*, *SDHC*, *SDHD*, and *VHL* was investigated by using multiplex ligation-dependent probe amplification (MLPA).

DNA sequencing

DNA was enriched from blood by the DNeasy kit from Qiagen (Qiagen, Hilden, Germany) using the Hamilton ML-Star pipetting robot (Hamilton, www.hamiltonrobotics.com/).

The primers, covering coding regions in *RET* gene (Accession number: NM_020975) exon 10, 11, 14, and 16 and all coding regions of the *VHL* (Accession number: L15409), *SDHB* (Accession number: NM_003000), and *SDHD* (Accession number: NM_003002) genes were designed using ExonPrimer (http://ihg.gsf.de/ihg/ExonPrimer.html) or Primer Express® Software v2.0 (Applied Biosystems), and PCR reactions were set up using the automated workstation Biomek® FX (Beckman Coulter, www.beckmancoulter.com) and performed in 10-µl reactions according to standard procedures. PCR products for *VHL*, *SDHB*, and *SDHD* were cleaned using magnetic beads (AMPure, Agencourt, Bioscience Corporation, Beverly, MA) and for *RET* using the Qiagen MinElute PCR Purification kit (Qiagen), and sequenced using BigDye® Terminator v 3.1 Cycle Sequence Kit (Applied Biosystems) in 10-µl reactions according to manufacturer's protocol. The sequence PCR thermal profile for GC-rich

fragments (e.g., *VHL* exon 1) were modified to longer denaturation steps and increased number of cycles (i.e., 50 cycles). The sequence-PCR products were cleaned using magnetic beads (CleanSeq, Agencourt) and separated by gel electrophoresis on a 3730 DNA Analyzer (Applied Biosystems). Results were analysed using Sequencing Analysis v. 5.2 and SeqScape v.2.5 (Applied Biosystems).

MLPA—SDHB, SDHC, SDHD, and VHL

DNA enriched from blood (cf. DNA sequencing—*SDHB*, *SDHD*, and *VHL* section) was analyzed for exon deletions by MLPA for *SDHB*, *SDHC*, and *SDHD* (SALSA P226, MRC Holland, Amsterdam, The Netherlands) and *VHL* (SALSA P016-B2) and run according to the manufacturer's recommendations. Results were analyzed using the GeneMapper v.3.7 software (Applied Biosystems), and normalization was performed in R 2.9.2. For each case, the MLPA peak heights and peak areas were compared to three independent controls from Western Sweden.

Results

From 1958 to 2009, 256 patients with Pheo or PGL were registered in the NCR (Fig. 1). Of these, 127 had Pheo or abdominal PGL with sporadic presentation (mean age 52.5 years; 54% women). Twenty-four patients had hereditary or syndromic presentation (MEN2 $n = 13$, NF1 $n = 9$, VHL $n = 1$, Carney syndrome $n = 1$), 20 had extra-abdominal paragangliomas, and 1 patient with primary hyperparathyroidism was misclassified in the

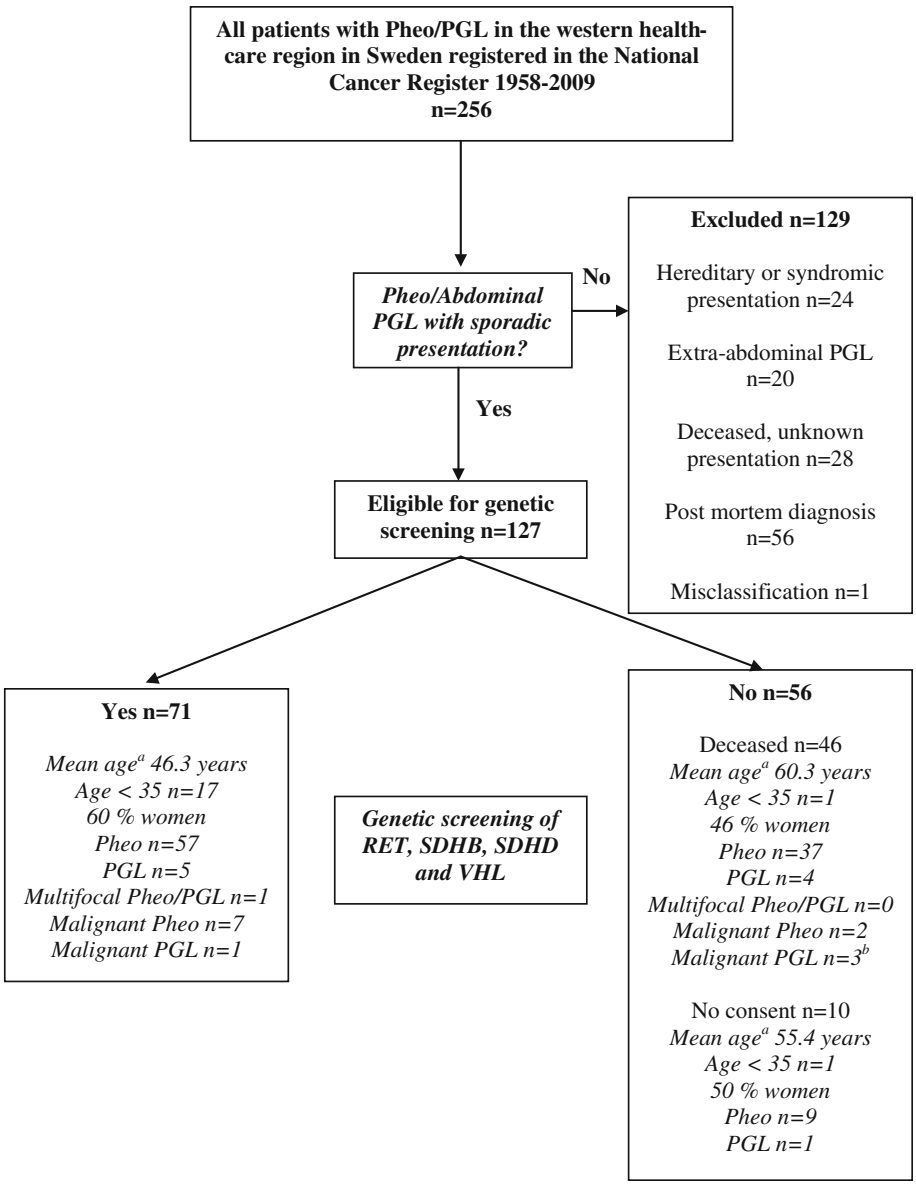

Fig. 1 Patients in Western Sweden with pheochromocytoma or paraganglioma 1958–2009. [a]Mean age at diagnosis. [b]The son of one of the patients had an *SDHB* mutation (c.418G>T) but no evidence of disease; the mutation status of the deceased father is unknown

Table 1 Characteristics of the detected patients with germline mutations and sporadic presentation of pheochromocytoma or abdominal paraganglioma

Age (years)[a], sex	Diagnosis	Malignant/ bilat/multif[b]	Secr	Mutation		LOVD ID[c]	FU (years)	Status
				c.DNA	Protein			
25, F	Pheo	No	E	*SDHB* c.716C>G	p.Ser239Cys	Submitted	26.0	NED
49, M	Pheo	No	NE	*SDHB* c.725G>A	p.Arg242His	SDHB_00004	15.7	NED
15, F	PGL	No	NE	*SDHB* c.IVS4+1G>A	Splice-site	SDHB_00047	28.1	NED
27, F	Pheo	No	E	*RET* c.1826G>A	p.Cys609Tyr	–	13	NED

Pheo pheochromocytoma, *PGL* paraganglioma, *Secr* secretory pattern, *E* epinephrine, *NE* norepinephrine, *FU* follow-up, *NED* no evidence of disease

[a] Age at diagnosis

[b] Malignant (metastases at presentation or during follow-up), bilateral or multifocal tumor

[c] Leiden Open Variation Database (http://chromium.liacs.nl/lovd_sdh/home.php?select_db=SDHB)

registry. Information on presentation was missing in 28 deceased patients (Pheo $n = 6$, PGL $n = 10$, malignant Pheo $n = 9$, malignant PGL $n = 3$; mean age 59.2 years, mean time from diagnosis to death 7.6 years). Fifty-six patients were diagnosed postmortem (Pheo $n = 39$, PGL $n = 5$, malignant Pheo $n = 12$).

Of the 127 patients with sporadic presentation, 81 were alive and were invited to the study, and 71 (88%) gave their consent and underwent testing. Forty-six of the 127 patients were dead; the mean survival time from diagnosis was 12 years. Patient characteristics are shown in Fig. 1.

Sequencing revealed one case with a missense mutation in the *RET*-gene, two cases with missense, and one case with a splice-site mutation in *SDHB* (Table 1). Six patients had single nucleotide polymorphisms in *SDHB*: c.18A>C (p.Ala6Ala) $n = 4$; c.24C>T (p.Ser8Ser) $n = 1$; c.487T>C (p.Ser163Pro) $n = 1$, Leiden Open Variants Database (LOVD) ID SDHB_00008, SDHB_00011, and SDHB_00038, respectively (http://chromium.liacs.nl/lovd_sdh/home.php?select_db=SDHB) [15]. No mutations were found in *SDHD* or *VHL*. No deletions or rearrangements were found by MLPA analysis.

The patient with a previously unrecognized *RET* p.Cys609Tyr mutation underwent a prophylactic thyroidectomy at age 40 years, 13 years after surgery for Pheo. At that time, she had no signs of recurrence of Pheo. On histopathological analysis, no C cell hyperplasia or medullary thyroid carcinoma was found. All three patients with *SDHB* mutations are alive without evidence of tumor recurrence or malignant development at 15.7, 26.0, and 28.1 years of follow-up, respectively (Table 1).

Discussion

In this population-based study of patients with Pheo or abdominal PGL with sporadic presentation registered in the National Cancer Registry for Western Sweden 1958–2009, the prevalence of germline mutations was 5.6%. Mutations were only seen in *RET* and *SDHB*. Notably, in the patients with *SDHB* mutations no evidence of malignancy was observed during a mean follow up of 23.3 years.

Differences in the reported frequency of germline mutations may reflect genetic differences in different populations/geographical areas and/or differences in patient selection. In the present study, 82% had isolated apparently sporadic single Pheo. Cascon et al. [10] reported 9% germline mutations in patients with a single Pheo/PGL and sporadic presentation, and only 2.3% in patients with a single apparently sporadic Pheo. In the present study, the susceptibility genes *RET*, *SDHB*, *SDHD*, and *VHL* were sequenced, and deletions were excluded in *SDHB*, *SDHC*, and *SDHD*-genes and *VHL*. The mutation detection methods used in this study are well-established techniques and have been used by other investigators in this context [9]. Whereas Sanger sequencing shows a >99.6% sensitivity for unidirectional analysis of heterozygous base substitutions [16], the MLPA technique is reported to show a sensitivity of approximately 92% [17]. The genetic testing methodology used is therefore not likely to explain the low prevalence of mutations found in this study. *SDHC*-mutation in apparently sporadic abdominal PGL seems rare [18].

Considering established risk factors for hereditary disease [7], tested patients were more likely to have germline mutations than patients who did not consent to the study and patients who had died before the study started, because tested patients were younger and more frequently had malignant disease. Still, a significant number of patients in the total cohort were deceased and a higher mutation frequency among these cannot be excluded. No systematic screening of relatives of living or deceased patients has yet been performed. However, a 39-year-old son of one deceased man with a malignant PGL has been diagnosed with an *SDHB* mutation (418 G>T, p.Val140Phe, LOVD

ID SDHB_00095). He has no evidence of disease. The mutation status of the father is unknown (Fig. 1).

Hereditary tumors occur at a younger age than sporadic tumors. Age <35 years at presentation is a risk factor for hereditary disease [7]. In this study, tested patients aged <35 years at presentation had a mutation frequency of 17.6%, compared to 1.9% in patients aged >35 years (Fig. 1; Table 1). It should be noted that three of four patients with germline mutations in the present study were younger than 30 years at presentation. In series of patients <20 years, one-third may be mutation carriers [9, 19].

SDHB carriers develop a malignant phenotype in 34.3–37.5% of the cases [20, 21]. In the present study, no patient with *SDHB* mutation had developed malignancy at presentation or during long-time follow-up (mean 23.3 years). Hypermethylation of the $P16^{INK4A}$ promotor has been associated with malignant phenotype in *SDHB* carriers [22], indicating stepwise genetic changes during the malignant transformation. To better tailor the follow-up of *SDHB* carriers, more information is needed on the impact of specific mutations and also how epigenetic alterations influence the phenotype.

Genetic analyses are time-consuming and costly. To minimize the number of analyses, most authors suggest a stepwise testing based on clinical data [8–10]. To speed up the process and cut costs, immunohistochemical analysis of SDHB protein expression in tumor tissue [23] has been used as a highly sensitive and specific screening tool to discriminate *SDH*-related from non-*SDH*-related tumours. Denaturing high performance liquid chromatography also has been used as a fast and relatively inexpensive screening method [24].

In this registry-based study, we found a frequency of germline mutations in patients with apparently sporadic pheochromocytoma and abdominal paraganglioma in Western Sweden of 5.6%. Differences in reported frequencies of germline mutations in patients with clinically sporadic Pheo/PGL may reflect geographical differences or patient selection.

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Conflict of interest The authors declare that they have no conflicts of interest.

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